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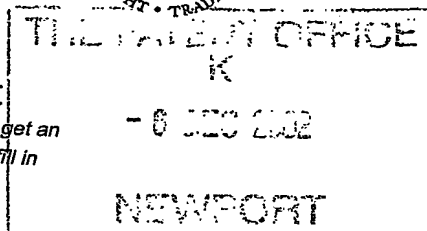
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1. Your reference

P32707-/CHO/BPU

2. Patent application number

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0228465.1

6 DEC 2002

3. Full name, address and postcode of the or of each applicant (underline all surnames)

The Queens University of Belfast
University Road
Belfast
BT7 1NN

Patents ADP number (if you know it)

5578786 005

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

A Method of Treating Disease

5. Name of your agent (if you have one)

Murgitroyd & Company

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Scotland House
165-169 Scotland Street
Glasgow
G5 8PL

Patents ADP number (if you know it)

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6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

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7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing
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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

Yes

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 - b) there is an inventor who is not named as an applicant, or
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Description 22

Claim(s)

Abstract

Drawing(s)

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent(Patents Form 7/77)

Request for preliminary examination and search(Patents Form 9/77)

Request for substantive examination (Patents Form 10/77)

Any other documents (please specify)

11. I/We request the grant of a patent on the basis of this application.

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Date

Murgitroyd & Company

6 December 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

Barry Purdy

0141 307 8400

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A Method of Treating Disease

1 The invention relates to a method of treating
2 disease. In particular, the invention relates to the
3 treatment of inflammatory and other diseases of the
4 type having a pathogenesis which involves endogenous
5 production of any of cytokines IL-12, IL 23 or IL
6 27.
7
8 Cytokines are a unique family of growth factors.
9 Secreted primarily from leukocytes, cytokines
10 stimulate both the humoral and cellular immune
11 responses, as well as the activation of phagocytic
12 cells. Cytokines secreted from lymphocytes are
13 termed lymphokines, whereas those secreted by
14 monocytes or macrophages are termed monokines. Many
15 of the lymphokines are also known as interleukins
16 (IL's), since they are not only secreted by
17 leukocytes, but are also able to affect the cellular
18 responses of leukocytes. Specifically, interleukins
19 are growth factors targeted to cells of
20 hematopoietic origin. One of the interleukins, IL-
21 12, is a pro-inflammatory cytokine interleukin. This
22 cytokine is predominantly secreted either as a $\alpha\beta$
23 heterodimeric form or as a $\beta\beta$ homodimeric form. Both
24 dimer forms bind the IL-12-receptor on target cells
25 but differ in the spectrum of biological activities
26 induced. The $\alpha\beta$ form is crucial for generation of
27 cell-mediated immunity against parasites, viruses
28 and bacteria, but contributes also to destructive
29 effects in pathogenesis of autoimmune diseases, e.g.
30 MS, RA and inflammatory bowel disease. The $\beta\beta$ form

1 has been shown to be instrumental in virus-induced
2 inflammation, and in excessive epithelial airway
3 inflammation seen in asthma. Thus, both forms of IL-
4 12 are disease-promoting factors in a variety of
5 conditions. Recently, two novel cytokines have been
6 discovered, named interleukin-23 and interleukin-27
7 that apparently belong to the IL-12 subclass of
8 cytokines based on structural relationships. Both
9 IL-23 and IL-27 share with IL-12 a typical
10 heterodimeric structure and are involved in a
11 similar array of immune responses.

12
13 Celebrex is a diaryl-substituted pyrazole. It is a
14 nonsteroidal anti-inflammatory drug (NSAID) that is
15 indicated for the treatment of osteoarthritis,
16 rheumatoid arthritis, for the management of acute
17 pain in adults for the treatment of primary
18 dysmenorrhea. The mechanism of action of CELEBREX is
19 believed to be due to inhibition of prostaglandin
20 synthesis, primarily via inhibition of
21 cyclooxygenase-2 (COX-2). Scientific literature
22 indicates that CELEBREX displays antitumor effects
23 by sensitizing cancer cells to apoptosis. A recent
24 paper has indicated that CELEBREX blocks the
25 endoplasmic reticulum (ER) Ca^{2+} -ATPases, and it has
26 been suggested that this Ca^{2+} perturbation may be
27 part of the signaling mechanism by which CELEBREX
28 triggers apoptosis. This Ca^{2+} perturbation effect
29 seems to be unique to CELEBREX and was not seen with
30 any of the other COX inhibitors (e.g. aspirin,
31 ibuprofen, naproxen etc.)

32

1 In a first aspect, the invention provides a method
2 of treating disease having a pathogenesis which
3 includes endogenous production of any of cytokines
4 IL-12, IL 23 or IL-27, the method comprising a step
5 of treating an individual with an endoplasmic
6 reticulum (ER) Ca^{2+} perturbation reagent.

7
8 In a second aspect, the invention provides the use
9 of an ER Ca^{2+} perturbation reagent in the manufacture
10 of a medicament for the treatment of disease having
11 a pathogenesis which includes endogenous production
12 of any of cytokines IL-12, IL-23 or IL-27.

13
14 In a third aspect, the invention provides the use of
15 an ER Ca^{2+} perturbation reagent for the treatment of
16 disease having a pathogenesis which includes
17 endogenous production of any of cytokines IL-12, IL-
18 23 or IL-27.

19
20 In a forth aspect, the invention relates to a method
21 of inhibiting the formation of one or more cytokines
22 in an individual, which method comprises the step of
23 treating an individual with ER Ca^{2+} perturbation
24 reagent. In one embodiment, the cytokines are
25 selected from IL-12, IL-23 and IL-27.

26
27 In a fifth aspect, the invention relates to the use
28 of an ER Ca^{2+} perturbation reagent to inhibit the
29 formation of one or more cytokines in an individual.
30 In one embodiment the cytokines are selected from
31 IL-12, IL-23 and IL-27.

32

1 In a preferred embodiment, the disease is an
2 inflammatory disease. More preferably, the disease
3 is a disease in which one or more endogenously
4 produced IL-12 forms play a disease promoting role.
5 Typically, the IL-12 forms are $\alpha\beta$ heterodimeric and
6 $\beta\beta$ homodimeric forms.

7
8 In one embodiment, diseases in which cyclooxygenase-
9 2 (COX-2) is reported to play a substantial disease
10 promoting role are disclaimed.

11
12 In one embodiment, the inflammatory disease is a
13 disease in which the endogenous production of one or
14 both of $\alpha\beta$ and $\beta\beta$ forms of IL-12 is known to lead to
15 disease in a COX-2 independent manner.

16
17 The invention also relates to a method of inhibiting
18 the production of one or more cytokines in an
19 individual in a post-translational manner, which
20 method comprises a step of treating an individual
21 with ER Ca^{2+} perturbation reagent.

22
23 Preferably, the disease is selected from the group
24 consisting of infectious diseases; bacterial
25 protozoal or virus-induced inflammation; epithelial
26 airway inflammation such as asthma; allergic
27 disease; autoimmune disease such as MS, RA and
28 Inflammatory Bowel Disease; and -all conditions in
29 which endogenously produced IL-12 α/β or $\beta\beta$ forms
30 are thought to play a disease-promoting role,
31 including:

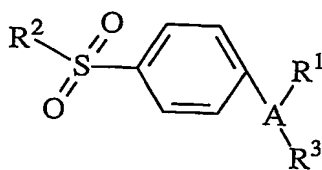
32

- 1 Pulmonary fibrosis
- 2 Pulmonary tuberculosis
- 3 Asthma
- 4 Sarcoidosis
- 5 Leprosy
- 6 Schistosomiasis
- 7 Lupus erythematosus
- 8 Lupus nephritis
- 9 Allograft rejection
- 10 Airway inflammation
- 11 Respiratory syncytial virus infection
- 12 Multiple sclerosis
- 13 Alzheimer's disease
- 14 Abortion (women with recurrent pregnancy loss)
- 15 Certain vaccines aimed at inducing TH2-type immune
- 16 responses
- 17 Experimental autoimmune myocarditis
- 18 Tuberculosis
- 19 Psoriatic arthritis
- 20 Rheumatoid arthritis
- 21 Osteoarthritis
- 22 Colonic inflammation (colitis)
- 23 Chron's Disease
- 24 Inflammatory bowel disease
- 25 Atopic dermatitis, AD (chronic stage)
- 26 Inflammatory skin disease
- 27 Insulin dependent diabetes mellitus Type I and II
- 28 Endotoxaemia
- 29 Exposure to organic dust
- 30 Periodontal diseases
- 31 Nephrotic syndrome
- 32 Hepatocellular damage in chronic hepatitis C

- 1 Primary biliary cirrhosis
- 2 Cancer patients (Various cancers, and various stages
- 3 in cancer that are typically accompanied with
- 4 dysregulated IL-12, IL-23 and/or or IL-27
- 5 production)
- 6 ANCA associated vasculitis and sepsis
- 7 Experimental crescentic glomerulonephritis
- 8 Atherosclerosis
- 9 Acute viral myocarditis
- 10 Autoimmune myocarditis
- 11 Experimental autoimmune myasthenia gravis
- 12 Uveitis (as Behret's disease)
- 13 Thyroiditis and Grave's disease
- 14 Thyroid autoimmune disease
- 15 Myelopathy (HTLV-I-associated myelopathy)
- 16 Symptomatic transient hypogammaglobulinaemia of
- 17 infancy (THI)
- 18 Selective IgA deficiency (SIgAD)
- 19 Schizophrenia
- 20 Primary malignant melanoma
- 21 Abdominal aortic aneurysm
- 22 Autoimmune thrombocytopenic purpura
- 23 Heatstroke
- 24 Meningococcal sepsis
- 25 Septic shock
- 26 Meningoencephalitis
- 27 Bacterial meningitis
- 28 Pregnancy
- 29 Pre-eclampsia
- 30 HELLP syndrome (hemolysis, elevated liver function
- 31 test and low platelet counts
- 32 Endometriosis

- 1 Acute pancreatitis
- 2 Lung fibrosis induced by silica particles
- 3 Scleroderma
- 4 Sjogren's syndrome
- 5 Ankylosis spondylitis
- 6 Hashimoto's thyroiditis
- 7 Autimmune anemias
- 8 Goodpasture's syndrome
- 9 Addison's disease
- 10 Autoimmune hemolytic anemia
- 11 Spontaneous infertility (sperm)
- 12 Poststreptococcal glomerulonephritis
- 13 Autoimmune neuritis (Guillian-Barré syndrome)
- 14 Sialadenitis
- 15 Brucellosis
- 16 Chickenpox and related viral diseases
- 17 Helicobacter Pylori-induced gastritis
- 18 Common Variable Immunodeficiency (CVI)
- 19
- 20
- 21 In one embodiment, the disease is a conditions
- 22 characterized by dysregulation of IL-12, IL-23 or
- 23 IL-27
- 24 production conferred by polymorphisms in their
- 25 respective genes, or by polymorphisms in genes
- 26 involved in the biological activation or signal
- 27 transduction pathway of these cytokines.
- 28
- 29 In one embodiment, the ER Ca^{2+} perturbation reagent
- 30 is selected from the compounds of Formula I:
- 31
- 32

1 Formula I



2
 3 wherein A is a substituent selected from partially
 4 unsaturated or unsaturated heterocyclyl and partially
 5 unsaturated or unsaturated carbocyclic rings;
 6 wherein R¹ is at least one substituent selected from
 7 heterocyclyl, cycloalkyl, cycloalkenyl and aryl,
 8 wherein R¹ is optionally substituted at a
 9 substitutable position with one or more radicals
 10 selected from alkyl, haloalkyl, cyano, carboxyl,
 11 alkoxycarbonyl, hydroxyl, hydroxyalkyl, amino,
 12 alkylamino, arylamino, nitro, alkoxyalkyl,
 13 alkylsulfinyl, halo, alkoxy and alkylthio;
 14 wherein R² is methyl or amino; and
 15 wherein R³ is a radical selected from hydrido, halo,
 16 alkyl, alkenyl, oxo, cyano, carboxyl, cyanoalkyl,
 17 heterocycloxy, alkyl, alkoxy, alkylthio, alkylcarbonyl,
 18 cycloalkyl, aryl, haloalkyl, heterocyclyl,
 19 cycloalkenyl, aralkyl, heterocyclylalkyl, acyl,
 20 alkylthioalkyl, hydroxyalkyl, alkoxycarbonyl,
 21 arylcarbonyl, aralkylcarbonyl, aralkenyl,
 22 alkoxyalkyl, arylthioalkyl, aryloxyalkyl,
 23 aralkylthioalkyl, aralkoxyalkyl, alkoxyaralkoxyalkyl,
 24 alkoxycarbonalkyl, aminocarbonyl,
 25 aminocarbonylalkyl, alkylaminocarbonyl, N-
 26 arylaminocarbonyl, N-alkyl-N-arylaminocarbonyl,
 27 alkylaminocarbonylalkyl, carboxyalkyl, alkylamino,
 28 N-aryl-amino, N-aralkyl-amino, N-alkyl-N-aralkyl-amino,
 29 N-alkyl-N-aryl-amino, aminoalkyl, alkylaminoalkyl, N-

1 arylaminoalkyl, N-aralkylaminoalkyl, N-alkyl-N-
2 aralkylaminoalkyl, N-alkyl-N-arylaminomethyl, aryloxy,
3 aralkoxy, arylthio, aralkylthio, alkylsulfinyl,
4 alkylsulfonyl, aminosulfonyl, alkylaminosulfonyl, N-
5 arylaminosulfonyl, arylsulfonyl, N-alkyl-N-
6 arylaminosulfonyl; or a pharmaceutically-acceptable
7 salt thereof.

8
9 In a preferred embodiment, the ER Ca^{2+} perturbation
10 reagent is selected from the compounds and
11 compositions described in US Patent 5,972,986,
12 Column 3, line 34 to Column 10, line 32. In a
13 particularly preferred embodiment, the ER Ca^{2+}
14 perturbation reagent is a diaryl- substituted
15 pyrazole marketed under the brand name CELEBREX
16 (Celecoxib). CELEBREX is chemically designated as 4-
17 [5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-
18 yl] benzenesulfonamide.

19
20 Alternatively, the ER Ca^{2+} perturbation reagent may
21 be thapsigargin or A23187.

22
23 The invention will be more clearly understood from
24 the following description of some embodiments
25 thereof, given by way of example only:

26
27 Recombinant cell lines that secrete various forms of
28 IL-12 under control of tightly regulated promoters
29 were generated. It was observed that treatment of
30 these cell lines with an ER Ca^{2+} perturbation reagent
31 such as thapsigargin inhibited secretion of both the
32 $\alpha\beta$ and $\beta\beta$ forms of IL-12. Given the Ca^{2+} perturbation

1 activity of CELEBREX, this compound was also tested
2 on assembly of IL-12, and found that it exerts a
3 similar inhibitory effect on the secretion of the $\alpha\beta$
4 and $\beta\beta$ forms of IL-12. There is a total block in the
5 secretory production of both dimer forms of IL-12,
6 and maximal effects are obtained with the normal
7 physiological working concentration of CELEBREX in
8 the absence of any apparent toxic effects as
9 measured with the MTT assay. These affects are
10 conferred in a post-transcriptional and post-
11 translation manner as there is no effect on mRNA of
12 IL-12. Though the precise mechanism by which
13 CELEBREX exerts these effects is still under
14 investigation, and without being bound by theory,
15 evidence has been produced to support a Ca^{2+} -
16 dependent disturbance in the folding pathway of IL-
17 12 due to impaired activity of certain chaperones in
18 the ER.

19
20 The inhibitory effect of CELEBREX on formation of
21 the $\alpha\beta$ and $\beta\beta$ forms of IL-12 in vitro indicates that
22 this drug is of interest for the treatment of
23 inflammatory conditions in which endogenous
24 production of these IL-12 forms is known to lead to
25 disease in a COX2-independent manner, including MS,
26 IBD, virus-induced inflammation and asthma.

27
28 IL-12 is a member of a family of cytokines that
29 includes two recently discovered members IL-23 and
30 IL-27. All of these cytokines have a typical
31 heterodimeric structure and display an array of both
32 overlapping and distinct activities. It is thought

1 that also IL-23 and IL-27 may contribute to
2 destructive inflammation in various conditions.
3 Given the similar subunit assembly configuration of
4 these 3 cytokines, it is likely that CELEBREX will
5 exert similar inhibitory effects on assembly of IL-
6 23 and IL-27. If confirmed, this would imply that a
7 single drug could be used for the simultaneous
8 inhibition of 3 cytokines. Most anti-cytokine drugs
9 work by inhibiting transcription of mRNA. To our
10 knowledge this is the first demonstration of a drug
11 that inhibits cytokine formation in a post-
12 translational manner on the level of folding and
13 secretion of the protein, i.e. by perturbation.

14

15 Experimental methods

16

17 Materials. Celecoxib (Celebrex) was obtained from
18 Hefei Sceneri Chemical Co.; thapsigargin was
19 obtained from Calbiochem and A23187 from Sigma.

20

21 Cell culture. HEK293 IL-12 β/β and α/β producing
22 cell lines were maintained in a CO₂ incubator at 37
23 °C (5% CO₂). Cells were cultured in DMEM medium
24 supplemented with 10% foetal bovine serum.

25

26 Transfection and production of stable cell lines.

27 Human embryonic kidney cells previously transfected
28 with a pVgRXR construct that encodes a functional
29 human retinoid X/ecdysonic receptor, were obtained
30 from Invitrogen (EcR-293). Cassettes coding for the
31 human IL-12 α and β -chains were amplified from the
32 respective full-length cDNAs and fused with a C-

1 terminal hexahistidine-tag (H_6). These were
2 introduced in expression vectors and transfected
3 with FuGENE (Boehringer Mannheim) into ECR-293
4 cells. Clones were selected with the antibiotics
5 Zeocin (400 μ g/ml) and G418 (600 μ g/ml). Protein
6 expression was induced with 5 μ M Ponasterone A
7 (Ecdysone analog).
8

9 Capture of α/β - and β/β -H6-chaperone complexes on
10 Ni^{2+} -NTA.

11 Following induction with Ponasterone A, cells were
12 lysed. - α/β and β/β - H_6 -chaperone complexes were
13 captured on Ni^{2+} -NTA agarose. The gel was washed 5
14 times with buffer A (100mM NaH_2PO_4 , 10mM TrisHCl, 8M
15 urea, pH 6.3), and elution was carried out with
16 buffer B (same as Buffer A, but pH 4.3). Complexes
17 were boiled in SDS loading solution + DTT. Proteins
18 were separated by 4-15% SDS-PAGE and transferred to
19 PVDF membranes. Detection was carried out using
20 anti-p35 antibody G161-566.14 (Pharmlingen).
21 Membranes were stripped and re-probed successively
22 with anti-chaperone antibodies (α -CRT, α -Grp78, α
23 -Grp94 & α -CNX; StressGen).
24

25 Treatment with inhibitors.

26 Cells were cultured in 12 well plates with a density
27 of 10^5 cells per well. Cells were treated with
28 reagents at different concentrations for two hours
29 and afterwards the cells were induced with 5 μ M of
30 Ponasterone A to produce the β/β and α/β chains.
31 Inhibitors were added to the culture medium of
32 induced cells at the following concentrations:

1 between 10 μ M and 100 μ M for celecoxib, between 0.1
2 to 30 μ M for A23187 and between 0.5 to 45 μ M for
3 thapsigargin. Medium was collected and lysates
4 prepared after 16 hours of induction.

5
6 Western Blot and immunodetection of IL-12 β/β and
7 α/β .

8 Culture medium, lysates and immunoprecipitated
9 fractions were mixed with non-reducing or reducing
10 loading solution and were subjected to 4-15% SDS-
11 PAGE. Proteins were transferred from gels to PVDF
12 membranes by electroblot. Membranes were blocked in
13 2% casein in TBS buffer. Immuno-detection was
14 performed with the mouse monoclonal anti α -chain
15 antibody purchased from PharMingen (G161-566) and
16 the mouse monoclonal anti β -chain antibody purchased
17 from Abcam (1-2A1). The secondary antibody was goat
18 anti-mouse IgG HRP-conjugated obtained from Jackson
19 Immunoresearch. The detection of IL-12 forms was
20 carried out using the ECL-plus kit from Amersham-
21 Pharmacia.

22 Cytotoxicity test.

23 The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl
24 tetrazolium bromide) reagent was use to determinate
25 the cytotoxicity level after treatment of cells with
26 the inhibitors. The test was performed in 96 well
27 plates to which different concentrations of
28 inhibitors were added. After two hours the cells
29 were induced with Ponasterone A. The MTT reagent was
30 added to the cells after 16 hours of induction and
31 the absorbance measurement was carried out in a
32 spectrophotometer at 570 nm.

1

2 Experimental findings

3

4 IL-12 is a secretory protein. Secretory proteins are
5 defined as proteins that are released by cells into
6 the extracellular milieu, and that exert their
7 biological activity by binding onto a specific
8 membrane receptor of target cells. 'Folding' (i.e.
9 generation of a correct three-dimensional structure)
10 of secretory proteins, such as IL-12, typically
11 occurs in a membrane-surrounded cell organelle,
12 named the endoplasmic reticulum (ER). The ER is
13 specifically enriched in chaperones, thioredoxin-
14 type isomerases and proteins involved in
15 glycosylation pathways. An important role of these
16 factors is to assist in ensuring correct folding of
17 secretory proteins during their transit in the ER
18 prior to their secretion into the extracellular
19 milieu. Improperly folded secretory proteins are
20 generally retained in the ER and subsequently
21 degraded by proteases and components of the
22 cytosolic proteasome. It was hypothesised that the
23 use of selected pharmacological agents that
24 interfere with the proper functioning of 'folding'-
25 assisting factors in the ER could be used to inhibit
26 proper folding, and, hence, secretion of IL-12.
27 As a first step, different tightly controlled
28 ecdysone-inducible recombinant cell lines expressing
29 functional C-terminally hexahistidine-tagged IL-12
30 α/β (heterodimer) and IL-12 β/β (homodimer) chains
31 were developed. The use of such recombinant cell
32 lines alleviates some of the problems related to the

1 use of natural producer cells of IL-12 (e.g.
2 restricted availability, lack of reproducibility
3 etc). These recombinant cell lines were used as a
4 means to study the processes that determine
5 regulation of folding, assembly and secretion of IL-
6 12 homo- and heterodimers. The following inhibitors
7 were used: (i) thapsigargin (an ER Ca^{2+} -ATPase
8 inhibitor), and (ii) the ionophore A23187 and (iii)
9 celecoxib (a putative ER Ca^{2+} perturbing reagent),
10 each over a wide range of concentrations.

11 Following a 16-hr treatment of cells with these
12 inhibitors, culture medium was collected and the
13 presence of secreted IL-12 forms was detected by
14 means of non-reducing SDS-PAGE and western
15 immunoblot. It was found that neither the α/β nor
16 the β/β dimer forms of IL-12 were present in the
17 culture medium of cells treated with thapsigargin
18 when this was added over a concentration range of
19 0.1 μM to 15 μM . The amount of extracellularly
20 secreted IL-12 dimer forms produced by thapsigargin-
21 treated cells was <5% of that produced by untreated
22 cells (maximal suppression was observed for all
23 concentrations of thapsigargin greater than or equal
24 to 0.1 μM). Similarly, the calcium ionophore A23187
25 suppressed formation of secreted IL-12 dimer forms
26 when it was used over a concentration range of 0.1
27 μM to 30 μM , with maximal suppression (>95% compared
28 to untreated cells) from 1 μM . Toxicity conferred by
29 these inhibitors over the test period of 16 hr as
30 measured with the MTT test was observed for
31 concentrations of thapsigargin >5-10 μM and for
32 concentrations of A23187 >10 μM . Thus, the maximal

1 suppression of secreted IL-12 dimer production is
2 achieved at an inhibitor concentration at which
3 toxic effects are totally absent, showing that both
4 IL-12-suppressive and cell-toxic effects conferred
5 by these inhibitors are independent. Secretion of
6 IL-12 α and β monomer forms was suppressed by
7 neither thapsigargin nor A23187.

8
9 Both thapsigargin and A23187 are likely to exert
10 these effects by decreasing the concentration of Ca^{2+}
11 in the ER. It is likely that the resulting
12 suboptimal concentration of Ca^{2+} in the ER blocks the
13 activity of Ca^{2+} -dependent chaperones and folding-
14 assisting proteins involved in the dimer formation
15 of IL-12. Since Celecoxib is thought to disturb the
16 Ca^{2+} concentration in the ER in much a similar way to
17 the mode of action of thapsigargin, it was
18 investigated whether this compound can be used to
19 suppress production of secreted IL-12 dimer forms.
20 Celecoxib was dissolved in DMSO and added to
21 recombinant HEK293 cells over a concentration range
22 from 10 μM to 100 μM . As a control DMSO-only treated
23 cells were used. Celecoxib concentrations were
24 chosen on the basis of available literature data,
25 and coincide with optimal activity of the compound
26 in various cell-based systems. Two hours later cells
27 were induced with Ponasterone A to produce IL-12 α/β
28 or β/β dimer forms. After 16 hrs of additional
29 incubation, culture medium was collected and
30 assessed for the presence of IL-12 dimer forms by
31 means of non-reducing SDS-PAGE and immunoblot. This
32 showed that Celecoxib suppressed production of

1 secreted IL-12 β/β homodimers by >95% when used at a
2 concentration equal to or larger than 30 μM ; and of
3 secreted IL-12 α/β heterodimers by >95% when used
4 at a concentration equal to or larger than 10 μM .
5 Secretion of IL-12 α and β monomer forms was not
6 suppressed by Celecoxib. Toxicity as measured with
7 the MTT assay was visible when cells were treated
8 for 16 hrs with a concentration of Celecoxib equal
9 to or larger than 100 μM .

10 The mechanistic basis of this effect was
11 investigated by analysing intracellular dimer
12 formation in cells treated with Celecoxib. Cells
13 were treated with Celecoxib and induced with
14 Ponasterone A as described above. After 16 hrs,
15 cells were lysed with Triton-X-100-containing
16 buffer, and lysates were submitted to non-reducing
17 SDS-PAGE and immunoblotted. IL-12 dimer forms were
18 present in the cell lysates of both untreated cells
19 and in cells treated with Celecoxib, and no
20 difference was observed in the respective amounts.
21 All data taken together, this suggests that
22 Celecoxib blocks secretion of IL-12 dimer forms by a
23 mechanism that involves intracellular retention of
24 preformed IL-12 dimer forms, and not by inhibition
25 of dimer formation.

26 It was then investigated whether Ca^{2+} -dependent
27 chaperones are possibly involved in the effects
28 conferred by Celecoxib. In view of the results
29 detailed above, it was hypothesized that the
30 disturbance of the Ca^{2+} -balance in the ER by
31 Celecoxib (i) is unlikely to block interaction of
32 IL-12 with chaperones during dimer formation (as

1 formation of the intracellular dimer is
2 uncompromised), but (ii) is likely to prevent the
3 release of Ca^{2+} -dependent ER chaperones from IL-12
4 dimer forms. The resulting complex of IL-
5 12/chaperones is then possibly retained in the ER
6 through interaction with a receptor in the ER that
7 specifically interacts with the KDEL sequence
8 present in most chaperones. To investigate this
9 hypothesis, cells were treated with Celecoxib for 2
10 hours, induced with Ponasterone A, and incubated for
11 a further 16 hours. Cells were lysed, and
12 intracellular IL-12 was immunoprecipitated from the
13 lysates by means of Ni^{2+} -NTA-agarose that binds the
14 C-terminal hexahistidine sequence. Purified
15 immunoprecipitates were submitted to reducing SDS-
16 PAGE and blotted onto PVDF membranes. These
17 membranes were probed for the presence of various ER
18 chaperones by incubating with antibodies specific
19 for calreticulin, Grp78/BiP and Grp94. This showed
20 that the amount of calreticulin - but not of Grp78
21 and Grp94 - associated with IL-12 dimer forms was
22 significantly increased in cells treated with
23 Celecoxib compared to untreated cells. The amount of
24 calreticulin co-immunoprecipitated with IL-12 from
25 Celecoxib-treated cells was at least 5 times larger
26 than that in control cells.

27 The present data demonstrates that Celecoxib
28 efficiently suppresses secretion of IL-12 α/β and
29 β/β dimer forms by a post-transcriptional and post-
30 translational mechanism that involves Ca^{2+} -dependent
31 intracellular retention of IL-12 dimers. Maximal IL-
32 12-suppressive effects are observed at a

1 physiological Celecoxib concentration in the absence
2 of any obvious toxic effects.

3

4 For oral administration, the medicament according to
5 the invention may be in the form of, for example, a
6 tablet, capsule suspension or liquid. The medicament
7 is preferably made in the form of a dosage unit
8 containing a particular amount of the active
9 ingredient. Examples of such dosage units are
10 capsules, tablets, powders, granules or a
11 suspension, with conventional additives such as
12 lactose, mannitol, corn starch or potatoes starch;
13 with binders such as crystalline cellulose,
14 cellulose derivatives, acacia, corn starch or
15 gelatins; with disintegrators such as corn starch,
16 potato starch or sodium carboxymethyl-cellulose;
17 and with lubricants such as talc or magnesium
18 stearate. The active ingredient may also be
19 administered by injection as a composition wherein,
20 for example, saline, dextrose or water may be used
21 as a suitable carrier.

22

23 For intravenous, intramuscular, subcutaneous, or
24 intraperitoneal administration, the compound may be
25 combined with a sterile aqueous solution which is
26 preferably isotonic with the blood of the recipient.
27 Such formulations may be prepared by dissolving
28 solid active ingredient in water containing
29 physiologically compatible substances such as sodium
30 chloride, glycine, and the like, and having a
31 buffered pH compatible with physiological conditions
32 to produce an aqueous solution, and rendering said

1 solution sterile. The formulations may be present in
2 unit or multi-dose containers such as sealed
3 ampoules or vials.

4
5 If the inflammatory disease is localized in the G.I.
6 tract, the compound may be formulated with acid-
7 stable, base-labile coatings known in the art which
8 began to dissolve in the high pH intestine.

9 Formulations to enhance local pharmacologic effects
10 and reduce systemic uptake are preferred.

11

12 Formulations suitable for administration
13 conveniently comprise a sterile aqueous preparation
14 of the active compound which is preferably made
15 isotonic. Preparations for injections may also be
16 formulated by suspending or emulsifying the
17 compounds in non-aqueous solvent, such as vegetable
18 oil, synthetic aliphatic acid glycerides, esters of
19 higher aliphatic acids or propylene glycol.

20

21 Formulations for topical use include known gels,
22 creams, oils, and the like. For aerosol delivery,
23 the compounds may be formulated with known aerosol
24 excipients, such as saline and administered using
25 commercially available nebulizers. Formulation in a
26 fatty acid source may be used to enhance
27 biocompatibility. Aerosol delivery is the preferred
28 method of delivery for epithelial airway
29 inflammation.

30

31 For rectal administration, the active ingredient may
32 be formulated into suppositories using bases which

1 are solid at room temperature and melt and dissolve
2 at body temperature. Commonly used bases include
3 cocoa butter, glycerinated gelatin, hydrogenated
4 vegetable oil, polyethylene glycols of various
5 molecular weights, and fatty esters of polyethylene
6 stearate.

7
8 The dosage form and amount can be readily
9 established by reference to known inflammatory
10 disease treatment or prophylactic regimens. The
11 amount of therapeutically active compound that is
12 administered and the dosage regimen for treating a
13 disease condition with the compounds and /or
14 compositions of this invention depends on a variety
15 of factors, including the age, weight, sex and
16 medical condition of the subject, the severity of
17 the disease, the route and frequency of
18 administration, and the particular compound
19 employed, the location of the inflammatory disease,
20 as well as the pharmacokinetic properties of the
21 individual treated, and thus may vary widely. The
22 dosage will generally be lower if the compounds are
23 administered locally rather than systemically, and
24 for prevention rather than for treatment. Such
25 treatments may be administered as often as necessary
26 and for the period of time judged necessary by the
27 treating physician. One of skill in the art will
28 appreciate that the dosage regime or therapeutically
29 effective amount of the inhibitor to be
30 administered may need to be optimized for each
31 individual. The pharmaceutical compositions may
32 contain active ingredient in the range of about 0.1

1 to 2000mg, preferably in the range of about 0.5 to
2 500mg and most preferably between about 1 and 200
3 mg. A daily dose of about 0.01 to 100mg/kg body
4 weight, preferably between about 0.1 and about
5 50mg/kg body weight, may be appropriate. The daily
6 dose can be administered in one to four doses per
7 day.

8

9 The invention is not limited to the embodiments
10 hereinbefore described which may be varied in detail
11 without departing from the invention.

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PCT Application
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